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Type 1 metabotropic glutamate receptors (mGlu1) trigger the gating of GluD2 delta glutamate receptors

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Editor: Barbara Pauly

1st Editorial Decision 01 May 2013

Thank you very much for the submission of your research manuscript to our editorial office. We have now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I will only repeat the main points here. You will see that the reviewers appreciate the interest of your findings and are, in principle, supportive of publication of your study in our journal. However, they also point out aspects of your study that would need to be further strengthened before publication. For instance, Referee 2 states that it should be tested whether D-serine and Pyr3 inhibit the GluD2 currents. Along similar lines, referee 3 points out that it should be tested whether L-glutamate activates the currents. This referee also states that the data on the effects of GluD2 on mGlu1 currents would need to be more carefully presented and must include baseline traces. This reviewer and referee 2 also point out several instances in which further clarifications or textual changes seem to be warranted. Both referees 1 and 3 also ask for clear statements on the statistical significance of the results. Finally, we think that the request of reviewer 2 to elucidate how mGlu1 gates GluD2 is beyond the scope of the current study and we would therefore not make this a prerequisite for publication. Obviously, if you already have data at hand that go in this direction you are very welcome to include them in the revised version.

Given these evaluations, the reviewers constructive comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the reviewers should be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. If you feel that this period is insufficient for a successful submission of your revised manuscript I can potentially extend this period slightly. Also, the length of the revised manuscript should not exceed roughly 29,000 characters (including spaces and references). If you feel that the additional data requested by the reviewers would make the manuscript too long you may consider including some peripheral data in the form of Supplementary information. However, Materials and Methods essential for the repetition of the main experiments should not be displayed as supplementary information only.

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We also welcome the submission of cover suggestions or motifs that might be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

GluD1 and GluD2 are homologous of other ionotropic glutamate receptor subunits yet there is currently no evidence that native wild type GluD1 or GluD2 act as functional ion channels although a channel pore mutant form of GluD2 (lurcher) can lead to ion currents. Recent work identifies the metabotropic/GPCR mGluR1 as binding partner for GluD2 and indicates that this complex might recruit current activity via TrpC channels.

In the current work the authors provide for the first time clear evidence that the non-mutant wild type GluD2 conducts current upon activation of mGluR1 when reconstituted in HEK293 cells. These findings are supported by a point mutation that blocks the pore and pharmacological inhibition in HEK cells and in ex vivo acute cerebellar slices by GluD2 mutants and again drug inhibition.

Major concern:

Although all experiments appear technically sound and well planned it is imperative that the number of independent experiments are given for each figure.

Modest concern:

Methods should be described in some more detail

Minor concern:

Fig. 3E: legend for neurexin and cerebellin symbols is inverted-neurexin is a presynaptic integral membrane protein and cerebellin and extracellular protein not vice versa as it currently is implied.

Referee #2:

mGlur1 and GluD2 are both present postsynaptically in parallel fiber -Purkinje cell synapses in the cerebellum. Knockout mice lacking mGluR1 or GluD2 have similar phenotypes characterized by ataxia and deficient LTD and the two proteins have been reported to exist in the same molecular complex together with protein kinase C -gamma and TRPC3 channels. Earlier studies indicate that mGluR1 activation triggers slow EPSC in Purkinje cells which are mediated by TRPC3 channels and modulated by GluD2 (e.g., Hartmann et al., 2008; Kato et al., 2012). No ligand-gated channel activity has been demonstrated for GluD2, and LTD phenotype in GluD2 knockout can be rescued by a channel-negative GluD2 mutant indicating that at least some important functions of GluD2 are independent of ion channel activity. The present manucript of Ady and coworkers suggests, surprisingly, that GluD2 channels do contribute to mGluR1-dependent slow EPSCs in Purkinje cells, and furthermore, that GluD2 channels are in fact gated by mGluR1. The manuscript is highly interesting and is written in a concise and clear manner and the conclusions are generally supported by the data shown. The experiments appear carefully performed and controlled. There are, however, some unclear points which need to be addressed.

1. The proposed gating of GluD2 channels by mGluR1 lacks a plausible mechanism. An interaction independent of modulating TRPC currents is mainly supported by HEK 293 cell experiments. HEK cells could be used to analyze the requirements for the slow current triggered by DHPG in cells coexpressing wild-type (but not channel-negative). Is the current second messenger -dependent; i.e., do other DAG/IP3-generating GPCRs produce a similar GluD2-mediated current? Alternatively, a direct interaction between mGluR1 and GluD2 ligand-binding domains may be involved, although the slow time-course of the response may speak against this possibility. Do GluD2 and mGluR1 interact in HEK293 cells and is the interaction dependent of mGluR agonists (or D-serine)?

2. Relation of the current findings to the published evidence against functionally important ionotropic activity of GluD2 should be discussed.

3. What is the evidence against the (admittedly remote) possibility that wild-type GluD2 (but not GluD2 mutants) might act as a chaperone in mGluR1 maturation and transport to cell surface rather than mediator of mGluR1-activated currents?

4. Figure 1. Some obvious control experiments lacking: Does D-serine inhibit the slow current in HEK cells? Does Pyr3 inhibit the current?

5. Figure 3A. The figure item does not represent amino acids sequences but rather schematic structure of the two proteins (wt D2 and mutant); in addition, the meaning of the numbers (416, 731) on top of HO-Nancy structure is not made clear (residues flanking the deletion?)

6. Figure 3E. The cartoon shows (misleadingly) neurexin as a soluble protein and cerebellin as presynaptic membrane protein.

Referee #3:

In their manuscript Ady et al. build on the recently published observation that the orphan ionotropic glutamate receptor delta2 (= GluD2) can be found in a complex with the metabotropic glutamate receptor 1 (mGlu1) and offer in vitro (heterologous expression in HEK cells) and in vivo (mouse cerebellar slices) evidence for the gating of GluD2 by mGlu1.

The authors provide a potentially interesting set of data to address the currently hotly debated issue

of GluD2 function. For GluD2, so far mainly structural, scaffolding functions have been proposed, while there are only relatively few studies that champion the functionality of GluD2's integral ion channel. Here, the authors weigh in on the side of an ionotropic function of GluD2 and conclude that the metabotropic glutamate receptor mGluR1 gates GluD2.
While the experimental approach taken by the authors is straightforward and suitable to address the question at hand, the manuscript unfortunately suffers from a number of shortcomings, both in experimental design and description as well as graphical presentation of the data that make this study less useful and convincing than it potentially could be.

Major shortcomings:

1. The data appear considerably oversold, a problem that starts right with the title, which claims "delta2 glutamate receptors are gated by type 1 metabotropic glutamate receptors".
First, the casual reader will probably assume that "gating" means the direct triggering of channel opening, much like glutamate triggers AMPA receptors. However, there is no evidence for that, and in the Discussion the authors actually backpaddle by saying more cautiously that mGluR1 "triggers" the gating of GluD2. Second, the term "type 1 metabotropic glutamate receptors" could be mistaken to mean group I metabotropic glutamate receptors, which would include mGlu1 and mGlu5. However, only mGlu1 has been investigated, not mGlu5.

2. In the Results section on p. 3, line 3 from the bottom, two references are given ("2, 21") to studies that allegedly tried DHPG unsuccessfully as a putative agonist on HEK cell-expressed GluD2. However, none of these studies did any experiments with DHPG.

3. The authors use RS-DHPG as an mGlu1 agonist in their HEK cell experiments, but then switch to S-DHPG in Purkinje cells. No explanation is given why the agonist was changed from the racemate to the optically pure S-form, and what effect hat might have on the interpretation of the data.

4. Amazingly, none of the figure legends provide any information on the concentration of compounds used, nor do they state the number of repetitions of the experiments. Unfortunately, in most cases that information also cannot be found in the main text. Thus, some experiments cannot really be judged in a meaningful way. Examples include Fig. 1A, the concentration of NMDA and that of glycine, if co-applied (a detail which is not indicated); Fig. 1B, the concentration of NASPM; Figs. 2B and 2C, the concentration of NASPM; Fig. 2C, the concentration of serine; Figs. 3C and S1A, the concentration of AIDA; Fig. S1C, the concentrations of NBQX and D-APV.
The number of repetitions is not provided for experiments in Figs. 1A, 1D, 1E, and 1F.

5. Fig. 1A: Why are only sample traces provided with no bar graphs, as has been done in Fig. 1F? This would be much more informative, and none of the three recording conditions in Fig. 1A are repeated in Fig. 1F.

6. Fig. 1F: The proportions of wt and mutant GluD2 cDNAs in the co-transfection experiments are not given.

7. As a conclusion from Fig. 1F the authors state on p. 5 "Recordings in HEK293 corroborate the view that these channels are functional and they designate mGlu1 as their physiological activator." However, in order not to mislead the reader it should be made clear that neither this experiment nor any other in this study show that mGlu1 is a d i r e c t activator of GluD2. There could be one or several intermediate steps that lead from mGlu1 activation to GluD2 currents, and the authors do not even speculate about what these intermediate steps or what the mediators could be.

8. On p. 6 the authors mention data on non-transfected Purkinje cells to be present in Fig. 2E, but fail to show these data in that figure.

9. Given the conclusion of the authors that mGlu1 activation via DHPG in HEK cells or by PF stimulation in mouse cerebellar slices activates GluD2, one must assume that application of the physiological agonist L-glutamate will do the same. Why was that not tried? Given the cocktail of blockers the authors used, cross-stimulation of other pathways should not be a concern. It would be very reassuring if L-glutamate could activate the same current responses as the drug DHPG, which could have unknown and undesired side effects.

10. In Fig. 2B, why is there no recording trace in the absence of both NASPM and Pyr3? Without this "baseline" trace the alleged 35% reduction caused by Pyr3 cannot be appreciated. Furthermore, in both Fig. 2B and 2C labeling of the traces should be more precise, preferentially naming all compounds applied instead of using the "+" sign to indicate additions. It remains unclear, for example, if the Pyr3 trace in 2B was recorded in the presence of DHPG, and if the "serine" trace in 2C was recorded in the presence of both DHPG and NASPM, or only DHPG.
And what is the unlabeled (black) trace in Fig. 2C?

11. The interpretation of the immunohistochemistry results in Figs. 2D and 3B is complicated by the fact that they were obtained with an anti-GluD1/2 antibody (was that a mono- or polyclonal antibody?) that is not specific for GluD2, as it also recognizes GluD1.

12. In Fig. 2E, why does the PF-slow EPSC control curve (GFP) take twice as long to peak as the PF-slow EPSC curves shown in Figs. 2B and 2C? And do the GluD2V617R-transfected cells also contain GFP? If so, please add this information in the legend and figure.

13. In Fig. 3A, the cartoon of GluD2 wildtype wrongly shows the S2 region as located downstream of TMD3, which would put it intracellular.

14. In Fig. 3C, what does the red trace represent? And why was AIDA not added to the recordings from WT slices, only to HO slices?

15. In Fig. 3D, is that really a time-scale in the range of seconds?

16. In the Discussion the authors write
"In neurons, mGlu1, TRPCs, AMPA-Rs and GluD2 interfere with each others [13, 30]. Manipulating this latter could thus change the expression or location of the others and hereby reduce mGlu1 currents. However, our data in HEK293 cell line exclude this scenario and clearly designate GluD2s as novel mGlu1 currents carriers."
If I understand this argument correctly, they claim that manipulating GluD2 expression should not change the interacting proteins in HEK cells. I do not see how the authors can claim this without actually testing for the expression of these proteins in HEK cells.
Furthermore, what exactly do they mean when they characterize GluD2 as an "mGlu1 current carrier"?

17. On p. 8 the authors speculate on functional differences that other mGlu1 splice variants may provide. Why didn't they simply test those splice variants in their HEK cell expression system? Such an experiment would be straightforward and easy enough to do.

18. On p. 8, 2nd paragraph the authors mention "functional coupling" of mGlu1 and GluD2. It would have been nice if they had actually discussed the possible molecular basis of such coupling, or at least mentioned candidate coupling molecules.

19.On p. 9, 2nd paragraph the authors claim that GluD1 and GluD2 should have similar properties. However, a recent study (Orth et al., Eur. J. Neurosci., in press) comes to the opposite conclusion.

Minor shortcomings:

1. The manuscript suffers from a lack of correction by a native speaker, or at least by a good spell-checker program which would have caught the numerous typos. If further considered, a correction of wording and expressions urgently needs to be done, as occasionally the meaning of a statement is obscured or ambiguous, due to equivocal wording or, more often, to missing or inappropriate punctuation.

2. Hardly any of the many abbreviations used are explained. This should be rectified. Examples include RS-DHPG, S-DHPG, Pyr3, D-APV, NBQX, AIDA, EPSC, and PF.

3. Neither in the Methods section nor elsewhere in the manuscript is it stated from which species the

mGlu1, NR1A, and NR2B clones are derived. This is important as the frequently used rat clones would add another species to the two different expression systems already used, human HEK cells and mouse cerebellar slices. Thus, species-specific differences could potentially become an issue.

4. In the Methods section, p. 10, 1st paragraph, what is "precipitation method 26"?

5. The Supplementary Methods are merely a repetition of the Methods section of the main manuscript, with a few additional words here and there. Those could easily have been added to the regular Methods section. But see below.

Suggestion regarding the length:

Should shortening of the manuscript be required, much of the Method section could be transferred to Supplementary Information.

1st Revision - authors' response 15 September 2013

The referees have made very constructive comments, we would like to thank them for their time. We have undertaken new experiments to answer their concerns and we have made the modifications suggested. Please find hereafter our point-by-point answers.

In addition to the experiments requested by the referees, we also added immunolabelings showing that mGlu1, GluD2 and GluD2V617R reach the membrane when transfected in HEK cells, whatever their combination of expression (supplementary information). This indicates that all the constructs transfected express and locate at the membrane of HEK293 cells and that the mutant proteins GluD2V617R do not prevent mGlu1 trafficking to the membrane.

Point-by-point answers to the referees.

Referee #1:

Major concern: Although all experiments appear technically sound and well planned it is imperative that the number of independent experiments are given for each figure.

The numbers of independent cells and experiments used have been added in the main text (nb cells) were necessary and have been precised in the methods and supplementary methods (nb of experiments, p.7 suppl. methods)).

Modest concern: Methods should be described in some more detail.

This has been fixed, mainly in supplementary methods due to space limitation.

Minor concern: Fig. 3E: legend for neurexin and cerebellin symbols is inverted-neurexin is a presynaptic integral membrane protein and cerebellin and extracellular protein not vice versa as it currently is implied.

This error is now corrected.

Referee #2:

[...]

There are, however, some unclear points which need to be addressed.

1. The proposed gating of GluD2 channels by mGluR1 lacks a plausible mechanism. An interaction independent of modulating TRPC currents is mainly supported by HEK 293 cell experiments. HEK cells could be used to analyze the requirements for the slow current triggered by DHPG in cells coexpressing wild-type (but not channel-negative). Is the current second messenger -dependent; i.e., do other DAG/IP3-generating GPCRs produce a similar GluD2-mediated current? Alternatively, a direct interaction between mGluR1 and GluD2 ligand-binding domains may be involved, although the slow time-course of the response may speak against this possibility. Do GluD2 and mGluR1 interact in HEK293 cells and is the interaction dependent of mGluR agonists (or D-serine)?

We agree that this point is very important and will require clarification. However, at present, we do not have data concerning the mechanisms of coupling. Given the format of the present paper and the time required to do the corresponding experiments, we feel that this point of mechanistic would rather be the subject of a next study. The present study aims at establishing the existence of the coupling and making the corresponding demonstration.

2. Relation of the current findings to the published evidence against functionally important ionotropic activity of GluD2 should be discussed.

This is now discussed, (p. 8, last paragraph)

3. What is the evidence against the (admittedly remote) possibility that wild-type GluD2 (but not GluD2 mutants) might act as a chaperone in mGluR1 maturation and transport to cell surface rather than mediator of mGluR1-activated currents?

We performed additional experiments showing that mGlu1 efficiently reaches the cell membrane when expressed either alone or with WT GluD2 or GluD2V617R (figures S1-S3). In addition, using a different HO mutant, the HO-4j, in which GluD2s remain trapped in the endoplasmic reticulum, Kato et al (J. Neurosci 2012) showed that mGlu1s are still functional in the absence of GluD2.

4. Figure 1. Some obvious control experiments lacking: Does D-serine inhibit the slow current in HEK cells? Does Pyr3 inhibit the current?

We did the experiments and now show the results in figure 1. As expected, in mGlu1/GluD2 transfected HEK cells, D-serine inhibits the mGlu1 current and Pyr3 has no effect.

5. Figure 3A. The figure item does not represent amino acids sequences but rather schematic structure of the two proteins (wt D2 and mutant); in addition, the meaning of the numbers (416, 731) on top of HO-Nancy structure is not made clear (residues flanking the deletion?)

The first sentence of Figure 3 legend has been changed to: "WT and HO-Nancy schematic proteins. Amino-acid numbers flanking the deletion are indicated"..

6. Figure 3E. The cartoon shows (misleadingly) neurexin as a soluble protein and cerebellin as presynaptic membrane protein.

This error has been corrected.

Referee #3:

[…]

Major shortcomings:

1. The data appear considerably oversold, a problem that starts right with the title, which claims "delta2 glutamate receptors are gated by type 1 metabotropic glutamate receptors". First, the casual reader will probably assume that "gating" means the direct triggering of channel opening, much like glutamate triggers AMPA receptors. However, there is no evidence for that, and in the Discussion the authors actually backpaddle by saying more cautiously that mGluR1 "triggers" the gating of GluD2. Second, the term "type 1 metabotropic glutamate receptors" could be mistaken to mean group I metabotropic glutamate receptors, which would include mGlu1 and mGlu5. However, only mGlu1 has been investigated, not mGlu5.

We understand the possible confusion underlined by this referee. In the title, and further in the text, we indicate in brackets, immediately after "type 1 metabotropic glutamate receptors", the abbreviation "mGlu1".

We also modified the text anywhere it was necessary to insist on the fact that mGlu1 activation triggers the gating of GluD2.

Concerning our title, it takes into account the journal guidelines (no more than 100 characters) and the fact that we prefer to insist on GluD2 gating rather than on mGlu1 currents. However, if Referee N°3 and/or the editor feel that changing the title is necessary for the publication, we can propose: "Type 1 metabotropic glutamate receptors (mGlu1) trigger the gating of GluD2 glutamate receptors"

2. In the Results section on p. 3, line 3 from the bottom, two references are given ("2, 21") to studies that allegedly tried DHPG unsuccessfully as a putative agonist on HEK cellexpressed GluD2. However, none of these studies did any experiments with DHPG.

We acknowledge this was an error. We meant that DHPG does not activate GluD2, like the other agonists tested by others (the references referred to these papers). We removed these 2 references.

3. The authors use RS-DHPG as an mGlu1 agonist in their HEK cell experiments, but then switch to S-DHPG in Purkinje cells. No explanation is given why the agonist was changed from the racemate to the optically pure S-form, and what effect hat might have on the interpretation of the data.

In HEK cells the racemate (initially) and the S enantiomer (more recently) were used. The two compounds gave similar results so we pooled the data. In acute cerebellar slices only S-DHPG was used. We indicated all this in the supplementary information: "drugs" section (due to space limitation we could not add this to the main text).

4. Amazingly, none of the figure legends provide any information on the concentration of compounds used, nor do they state the number of repetitions of the experiments. Unfortunately, in most cases that information also cannot be found in the main text. Thus, some experiments cannot really be judged in a meaningful way. Examples include Fig. 1A, the concentration of NMDA and that of glycine, if co-applied (a detail which is not indicated); Fig. 1B, the concentration of NASPM; Figs. 2B and 2C, the concentration of NASPM; Fig. 2C, the concentration of serine; Figs. 3C and S1A, the concentration of AIDA; Fig. S1C, the concentrations of NBQX and D-APV.

We indicated the missing concentrations in the main text.

The number of repetitions is not provided for experiments in Figs. 1A, 1D, 1E, and 1F.

We now give the numbers in the text and give additional details in suppl. information ("Number of cells/experiments", p.7).

5. Fig. 1A: Why are only sample traces provided with no bar graphs, as has been done in Fig. 1F?

We added the bars to the figures

This would be much more informative, and none of the three recording conditions in Fig. 1A are repeated in Fig. 1F. 6. Fig. 1F: The proportions of wt and mutant GluD2 cDNAs in the co-transfection experiments are not given.

mGlu1 + GluD2 transfected HEK cells are also used in 1E, F. We added the quantity of plasmid transfected directly on figure 1F.

7. As a conclusion from Fig. 1F the authors state on p. 5 "Recordings in HEK293 corroborate the view that these channels are functional and they designate mGlu1 as their physiological activator." However, in order not to mislead the reader it should be made clear that neither this experiment nor any other in this study show that mGlu1 is a direct activator of GluD2. There could be one or several intermediate steps that lead from mGlu1 activation to GluD2 currents, and the authors do not even speculate about what these intermediate steps or what the mediators could be.

We agree with this remark. We do not have enough space to discuss possible intermediates. Such discussion would remain, anyway, very speculative at this point. However to clarify this point, we added to our discussion that that "none of our data suggest that [the coupling] involves a direct activation of GluD2 by mGlu1, intermediates can not be excluded" (p. 8).

8. On p. 6 the authors mention data on non-transfected Purkinje cells to be present in Fig. 2E, but fail to show these data in that figure.

Figure 2E shows the cells that have been transfected with GFP alone, as controls for GFP+V617RGluD2 transfected cells. Data about cells not transfected are illustrated in figure 2B. We modified the text to avoid erroneous interpretations.

9. Given the conclusion of the authors that mGlu1 activation via DHPG in HEK cells or by PF stimulation in mouse cerebellar slices activates GluD2, one must assume that application of the physiological agonist L-glutamate will do the same. Why was that not tried? Given the cocktail of blockers the authors used, cross-stimulation of other pathways should not be a concern. It would be very reassuring if L-glutamate could activate the same current responses as the drug DHPG, which could have unknown and undesired side effects.

We tried to activate mGlu1 with glutamate. However, glutamate induces very large inward currents, even in non-transfected HEK cells, or even in the presence of glutamate transporters inhibitors in addition to iGluRs inhibitors (in Purkinje cells). This large current masks any other currents and, thus, renders the data inconclusive. Given that DHPG by itself does not induce any current in HEK cells transfected with mGlu1 alone or with GluD2 alone or with mGlu1+NMDA, we think that it is reasonable to consider that DHPG has no significant side effects. In addition, given that DHPG currents as well as PF slow EPSCs are inhibited by AIDA, the currents

that we recorded are very likely mediated by mGlu1 activation. Finally, the fact that PF stimulation also triggers GluD2 currents supports the view that glutamate also activates the mGlu1-dependent GluD2 current.

10. In Fig. 2B, why is there no recording trace in the absence of both NASPM and Pyr3? Without this "baseline" trace the alleged 35% reduction caused by Pyr3 cannot be appreciated.

We added the traces requested to this figure.

Furthermore, in both Fig. 2B and 2C labeling of the traces should be more precise, preferentially naming all compounds applied instead of using the "+" sign to indicate additions. It remains unclear, for example, if the Pyr3 trace in 2B was recorded in the presence of DHPG, and if the "serine" trace in 2C was recorded in the presence of both DHPG and NASPM, or only DHPG. And what is the unlabeled (black) trace in Fig. 2C?

None of the recordings shown in figure 2 have been made using DHPG. Only synaptic, but not pharmacological, activation of mGlu1 has been performed (PF-slow EPSC). We modified the figure as requested and clarified the different experimental conditions used.

11. The interpretation of the immunohistochemistry results in Figs. 2D and 3B is complicated by the fact that they were obtained with an anti-GluD1/2 antibody (was that a mono- or polyclonal antibody?) that is not specific for GluD2, as it also recognizes GluD1.

We did new experiments with a different polyclonal antibody, which is specific for GluD2. We replaced our initial fig. 2D by the images that we obtained with this GluD2-specific antibody and moved the original figure to the supplementary information. The results are similar when using the two different antibodies suggesting that GluD1 has minor contribution to the labeling obtained with the anti-GluD1/2 antibody. We added the species and the poly- or monoclonal nature of the antibodies used in the supplementary methods (p.5 – 2 first sentences).

12. In Fig. 2E, why does the PF-slow EPSC control curve (GFP) take twice as long to peak as the PF-slow EPSC curves shown in Figs. 2B and 2C?

In our hands, the PF-slow EPSC kinetics are variable from cell to cell, whatever the genotype or manipulation we use. This is true for WT and HO-Nancy mice and also for Purkinje cells transduced with Sinbis virus, whatever the constructs used. Examining our data, it appears that we can neither make any correlation between these kinetics and, for example, the amplitude of the initial AMPA mediated PF-EPSC (before we add NBQX), the effects of Pyr3 or the position of the stimulating pipette. We have no explanation for this cell-to-cell variability. Our supplementary figure S4A displays an example of a PF-slow EPSC recorded in a WT Purkinje cell that also has slow kinetics. In fact, in about a quarter of the Purkinje cells we recorded, the PF-slow EPSCs kinetics is slower than in the others. In fig. 2E, the traces displayed are averages of PF-EPSCs recorded from different cells. In the GFP alone condition, two of these cells peaked around 600 ms which resulted in a slower averaged trace. By contrast, fig. 2B and 2C, show example of individual PF-slow EPSCs recorded from 2 different cells with faster kinetics. Such cells are more representative of kinetics we generally encountered in our experiments.

And do the GluD2V617R-transfected cells also contain GFP? If so, please add this information in the legend and figure.

Yes they do. This has been mentionned in the text (bottom of p.5 and of p.9; p.11 "virus injection") and on the figure.

13. In Fig. 3A, the cartoon of GluD2 wildtype wrongly shows the S2 region as located downstream of TMD3, which would put it intracellular.

We corrected this error.

14. In Fig. 3C, what does the red trace represent? And why was AIDA not added to the recordings from WT slices, only to HO slices?

The red trace is a representative PF-slow EPSC as can be recorded in the HO-Nancy Purkinje cells that display an mGlu1 EPSC. The effect of AIDA on PF-slow EPSCs being already known, the AIDA trace in a WT Purkinje cell is shown in the supplementary information (Fig. S4A).

15. In Fig. 3D, is that really a time-scale in the range of seconds?

This corresponds to currents induced by 30 sec bath application of 50 microM DHPG (see main text), which explains the slow kinetics of the current.

16. In the Discussion the authors write "In neurons, mGlu1, TRPCs, AMPA-Rs and GluD2 interfere with each others [13, 30]. Manipulating this latter could thus change the expression or location of the others and hereby reduce mGlu1 currents. However, our data in HEK293 cell line exclude this scenario and clearly designate GluD2s as novel mGlu1 currents carriers." If I understand this argument correctly, they claim that manipulating GluD2 expression should not change the interacting proteins in HEK cells. I do not see how the authors can claim this without actually testing for the expression of these proteins in HEK cells. Furthermore, what exactly do they mean when they characterize GluD2 as an "mGlu1 current carrier"?

We understand that our argumentation and its formulation were misleading so we changed the text for more clarity. P.7: " The presence of the dominant-negative or the HO-Nancy GluD2s could have changed the number of mGlu1s or TRPC3 [13, 30], thereby explaining the decrease of the mGlu1 current. This is very unlikely. GluD2s do not seem to behave as scaffold or auxiliary proteins [3, 13]. Moreover, the existence of a GluD2-dependent mGlu1 current in HEK293 cells that is inhibited by D-serine, NASPM and GluD2V617R but not Pyr3 shows that the mGlu1 current flows through GluD2s, and not through some other interacting channel."

17. On p. 8 the authors speculate on functional differences that other mGlu1 splice variants may provide. Why didn't they simply test those splice variants in their HEK cell expression system? Such an experiment would be straightforward and easy enough to do.

We agree that this is speculative. The present format of the paper is a report, so we feel that testing this hypothesis is rather the matter of another study. We removed this

part from the discussion and we now just mention this hypothesis as a possibility among others. p. 7: […]The respective contribution of TRPC1/3 and GluD2 may depend on the experimental conditions and/or on the splicing variants of mGlu1, as these latter vary among cerebellar regions [36]. These conditions remain to be clarified."

18. On p. 8, 2nd paragraph the authors mention "functional coupling" of mGlu1 and GluD2. It would have been nice if they had actually discussed the possible molecular basis of such coupling, or at least mentioned candidate coupling molecules.

At this point, none of our present data indicate any candidate coupling molecule. This interesting and important point therefore requires more investigation, i.e. more undetermined time before getting the answer. In the present study, we aimed at establishing the existence of the coupling by making the corresponding demonstration, which fits the format of a report.

19.On p. 9, 2nd paragraph the authors claim that GluD1 and GluD2 should have similar properties. However, a recent study (Orth et al., Eur. J. Neurosci., in press) comes to the opposite conclusion.

Orth et al. (Eur J Neurosci. 2013 May;37(10):1620-30) show that replacing the LBD of GluD1 with that of GluK2 results in glutamate-induced currents flowing through the GluD1 pore. Accordingly, Orth et al. state in the abstract that "the kainate receptor ligand binding domain renders GluD1 functional" and in the conclusion that "with the exception of the LBD, all receptor domains of both GluD1 and GluD2 are designed to and capable of sustaining true ligand-gated ion channel function". We have clarified this point by changing the sentence to: "The other delta family member GluD1 has 60% sequence homology with GluD2 [40] and is similarly endowed with a functional channel pore domain [21, 41]", in which reference 21 is the Orth et al. paper.

Minor shortcomings:

1. The manuscript suffers from a lack of correction by a native speaker, or at least by a good spell-checker program which would have caught the numerous typos. If further considered, a correction of wording and expressions urgently needs to be done, as occasionally the meaning of a statement is obscured or ambiguous, due to equivocal wording or, more often, to missing or inappropriate punctuation.

The manuscript has been thoroughly verified.

2. Hardly any of the many abbreviations used are explained. This should be rectified. Examples include RS-DHPG, S-DHPG, Pyr3, D-APV, NBQX, AIDA, EPSC, and PF.

PF and EPSC are detailed at their first use in the main text. Due to space limitation, we added the complete names of the chemical to the supplementary information.

3. Neither in the Methods section nor elsewhere in the manuscript is it stated from which species the mGlu1, NR1A, and NR2B clones are derived. This is important as the frequently used rat clones would add another species to the two different expression systems already used, human HEK cells and mouse cerebellar slices. Thus, species-specific differences could potentially become an issue.

mGlu1, NR1A and NR1B clones derive from the rat. However, their sequence homology with the mouse is very high: respectively 98.667%, 99.787% and 99.46%. We added this information to the supplementary methods p.2 "Plasmids and virus production" section.

4. In the Methods section, p. 10, 1st paragraph, what is "precipitation method 26"?

This error in quoting the reference has been corrected

5. The Supplementary Methods are merely a repetition of the Methods section of the main manuscript, with a few additional words here and there. Those could easily have been added to the regular Methods section.

But see below. Suggestion regarding the length: Should shortening of the manuscript be required, much of the Method section could be transferred to Supplementary Information.

We carefully modified the methods in the main text and in the supplementary information to address this issue.

2nd Editorial Decision 02 October 2013

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the two referees that were asked to assess it are now positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

1. Referee 3 indeed feels that the title should be changed to the alternative one you proposed in your point-by-point response. I agree that it reflects the data better and would therefore kindly ask you to change it.

2. This referee also feels that the differences in the PF-slow EPSC kinetics should be mentioned and, if possible, further discussed.

3. I have also taken the liberty of modifying the abstract slightly, as I feel that it reads a little easier now. Please make sure that I have not changed the meaning of it, though.

Suggested abstract:

The orphan GluD2 receptor belongs to the ionotropic glutamate receptor family but does not bind glutamate. Ligand-gated GluD2 currents have never been evidenced, and whether GluD2 operates as an ion channel has been a long-standing question. Here, we show that GluD2 gating is triggered by type 1 metabotropic glutamate receptors, both in a heterologous expression system and in Purkinje cells. Thus, GluD2 is not only an adhesion molecule at synapses but also works as a channel. This gating mechanism reveals new properties of glutamate receptors that emerge from their interaction and opens unexpected perspectives regarding synaptic transmission and plasticity.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORTS:

Referee #2 (Report):

My concerns and criticisms # 2-6 have been addressed in an adequate and satisfactory manner, but my main criticism (#1) remains. The claim of GluD1 being gated by mGluR1 strongly implies a direct mechanistic coupling, but this issue is left entirely open. However, irrespective of the mechanism, the presented findings are highly intersting and well deserve to be published, but the title and all mentions to gating should be toned down to be more consistent with the actual data.

Referee #3 (Report):

In the revised version of their manuscript and in the rebuttal, Ady et al. have satisfactorily addressed all the issues raised in my original review.

Regarding my comment listed as "Major shortcoming 1", I strongly urge the editor to insist on the use of the alternative title suggested in the rebuttal,
"Type 1 metabotropic glutamate receptors (mGlu1) trigger the gating of GluD2 glutamate receptors".
This title is much more precise than the original title "Delta2 glutamate receptors GluD2 are gated by type 1 metabotropic glutamate receptors mGlu1" which was also used in the revised manuscript.

Finally, regarding my comment listed as "Major shortcoming 12", while I understand that the authors really cannot explain the conspicuous differences in kinetics observed, they should at least acknowledge the fact and mention it somewhere in the manuscript instead of "hiding" the issue by remaining silent about it.

With these two stipulations I now can recommend the manuscript for publication.

2nd Revision - authors' response 08 October 2013

Thank you for you kind assistance during the review process and for having accompanied us in the revision of our paper.

As you and referee #3 suggested, I changed the title. It is now: "Type 1 metabotropic glutamate receptors (mGlu1) trigger the gating of GluD2 delta glutamate receptors".

I modified the abstract as you suggested.

I also added a few lines of discussion about the kinetics of the PF-EPSCs as requested by referee #3 in the manuscript (starting last line p.5) and a few lines of comments in the supplementary figure S4 legends (as this figure also displays a slow mGlu1 current trace) p.7 of the supplementary information.

I hope that these minor revisions answer your expectations.

Please, do not hesitate to contact me for any additional request.

Thank you for your time and consideration.

3rd Editorial Decision 10 October 2013

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

